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A solo long terminal repeat (LTR) of an endogenous retrovirus-like element (intracisternal A particle) present in the mouse MIPP gene promotes placenta-specific expression of a 1.2 kb message. We have previously shown that many mouse mammary preneoplasias and carcinomas ectopically express 2.2 and 5.6 kb MIPP-related mRNAs. We determined that the 3' ends of all MIPP transcripts, which are homologous to the kelch repeat motif, have the same sequence. PCR-based techniques were employed to clone the 5' end of the 2.2 kb transcript. Sequence analysis revealed a 1.7 kb open reading frame with an N-terminal BTB/POZ protein-protein interaction domain and six C-terminal kelch repeats. Using recombinant proteins from bacteria, we showed that MIPP co-sediments with microfilaments in vitro. Furthermore, MIPP and actin co-immunoprecipitate from MOD mouse mammary tumor cells. Western blotting with an antiserum we raised against recombinant MIPP protein indicates that only one MIPP protein, about 70 kDa, is translated in mouse mammary tumor cells, despite expression of two transcripts. Immunofluorescence has localized the protein to the cytoplasm, with a non-filamentous staining reminiscent of endoplasmic reticulum. We have transfected MIPP into normal mammary epithelial cells, and transformation studies are in progress to determine whether MIPP contributes to mammary tumorigenesis.

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FOREWORD

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Introduction

Intracisternal A particles (IAPs) are retrotransposons of which 1000 copies are endogenous to the haploid mouse genome (Lueders and Kuff, 1977). Like retroviruses, IAPs have gag, pol, and env sequences flanked on both ends by long terminal repeats (LTRs) (Aota et al., 1987; Mietz et al., 1987). The LTRs of IAPs carry the signals necessary for promotion, initiation, and polyadenylation of transcription (Lin and Lee, 1988; Lueders et al., 1984), and can also act as enhancers (Christy and Huang, 1988). The genetic regulatory mechanisms of IAP LTRs are applicable not only to IAP expression proper, but to that of nearby genes as well. A solo IAP LTR in the mouse IAP-promoted placental (MIPP) gene, for instance, promotes placenta-specific expression of a 1.2 kb message (Chang-Yeh et al., 1991). All strains of mice express placenta-restricted 2.2 and 4.4 kb MIPP-related mRNAs, but only mouse strains with the MIPP-associated LTR, such as BALB/c, express the 1.2 kb transcript (Chang-Yeh et al., 1993). The 2.2 and 4.4 kb transcripts have not been cloned before now, and their promoters are not known. The 1.2 kb MIPP mRNA contains an open reading frame (ORF) which putatively codes for a 202 amino acid protein with sequence homology to the C-terminal region of kelch family proteins (Chang-Yeh et al., 1991). This domain consists of five or six repeating units of 50 amino acids in most kelch-related proteins. However, only four repeats are present in the MIPP protein sequence derived from the 1.2 kb RNA. Therefore, this transcript might represent a truncated form of normal MIPP RNAs resulting from use of the LTR promoter. Kelch is a Drosophila protein which cross-links actin in the intercellular bridges (ring canals) connecting the cytoplasm of nurse cells with the oocyte (Robinson and Cooley, 1997; Xue and Cooley, 1993). In addition to the C-terminal repeats, it contains an N-terminal BTB/POZ domain, a protein-protein interaction domain found in many DNA-binding zinc-finger proteins and in several kelch family proteins (Albagli et al., 1995). Four kelch family proteins have demonstrated actin-binding activity (Eichinger et al., 1996; Hernandez et al., 1997; Robinson and Cooley, 1997; Sanders et al., 1996), and there is evidence, albeit indirect, to suggest that other kelch proteins associate with microfilaments (Itoh et al., 1999; Philips and Herskowitz, 1998; Varkey et al., 1995; Way et al., 1995). Despite the evidence that several kelch proteins bind microfilaments, for others there is evidence which suggests the contrary (von Bulow et al., 1995; Way et al., 1995; Wilson et al., 1997; Wolff et al., 1998).

One of the most frequently occurring abnormalities in BALB/c mouse mammary tumors and pre-neoplastic lesions, irrespective of etiology, is overexpression of IAPs relative to normal mammary gland (Asch and Asch, 1990; Asch et al., 1993). Since the MIPP 1.2 kb mRNA is promoted by an IAP LTR which is active in placenta, but otherwise normally inactive (Chang-Yeh et al., 1991), we hypothesized that MIPP might be expressed in BALB/c mouse mammary carcinomas (MMC). We have previously shown that BALB/c mouse mammary preneoplasias and carcinomas of several etiologies ectopically express 2.2 and 5.6 kb MIPP-related mRNAs (VanHouten et al., 1996). The 5.6 kb transcript is unique to the mammary lesions. MIPP messages were not detected in normal mammary gland from virgin, pregnant, and lactating mice. Additionally, in the mouse mammary tissues, IAP expression was found to correlate with MIPP expression. The ectopic expression of MIPP in the mammary tumors makes it a candidate for a novel oncogene in BALB/c mice. Therefore, one of the purposes of the proposed research is to determine whether the two MIPP mRNA species expressed in BALB/c mammary carcinomas are oncogenic. A goal of this research, therefore, is to clone and transfect the MIPP cDNAs into mammary epithelial cells for growth/tumorigenicity/invasiveness assays. Another goal of this research is to determine the function of MIPP protein (whether it is actin-binding like some other kelch family proteins).

Summary of Findings

The parallels between the expression of MIPP and IAPs suggested that the IAP LTR may play a role in transcriptional regulation of the gene. To address the question of whether activation of the solo LTR causes MIPP to be expressed in BALB/c mouse mammary tumors, a reverse transcriptase-polymerase chain reaction (RT-PCR) strategy was used. For this experiment, I assumed that all MIPP mRNAs use the same polyadenylation signal. RNA from a MIPP-expressing BALB/c mammary tumor, from normal mammary gland of a lactating mouse, and from placenta were reverse transcribed using an oligo-dT primer and Moloney murine

leukemia virus (MMLV) reverse transcriptase (RT). Two sets of PCR reactions were carried out on the cDNA from these three tissues using a 3' primer derived from the region containing the polyadenylation signal. One set of reactions used a 5' primer from the MIPP LTR, and the other set used a 5' primer downstream from the LTR. Using a 3' primer corresponding to a region near the polyadenylation signal, and 5' primer downstream from the U5 region of the LTR from the MIPP cDNA clone (17), one band of 881 bp was amplified from placenta and a D2 tumor. This band is the one expected to be amplified from the 1.2 kb placental transcript. However, using the same 3' primer with a 5' primer including 15 bases from the U5 sequences of the LTR, the anticipated band of 949 bp was amplified from placenta but no band was amplified from the D2 tumor. Therefore, LTR sequences are not present in the MIPP mRNA expressed in mouse mammary tumors. Furthermore, the size of the fragment amplified by the downstream (non-LTR) 5' primer was the same in the tumor as in placenta, and only one band was amplified from each tissue, despite the expression of multiple related transcripts. This indicates that all MIPP-related transcripts (1.2 kb, 2.2 kb, 4.4 kb, and 5.6 kb) have a common 3' end starting after the LTR. As expected, neither MIPP-specific primer set used in the RT-PCR amplified cDNA from normal mammary gland of a lactating mouse.

The high frequency of ectopic MIPP expression in both mouse mammary preneoplasias and tumors suggests that MIPP might be a new oncogene which contributes to at least some pathways of neoplastic progression in the BALB/c mouse mammary gland. To determine whether MIPP is a mammary oncogene, it is necessary to clone and characterize the transcripts expressed in the tumors. The effects of these clones on the *in vitro* and *in vivo* growth properties of mammary epithelial cells can then be analyzed. Several strategies have been employed with the aim of cloning the MIPP mRNAs found in BALB/c mammary carcinomas. Traditional cDNA library techniques using poly-A$^+$ RNA from a MIPP-expressing mouse mammary tumor and the ZAP-cDNA Gigapack II kit (Stratagene, La Jolla, CA) were tried first. A cDNA library was also made from a MIPP-expressing tumor using the SMART cDNA library synthesis kit (Clontech). The SMART oligonucleotide recognizes the 7-methylguanosine cap at the 5' end of eukaryotic mRNAs. When the reverse transcriptase (RT) reaches the 5' end of the mRNA, it switches templates to copy the SMART oligonucleotide. Long distance PCR is then used to amplify second strand cDNA. In these techniques, either oligo-dT or MIPP-specific oligonucleotides were used to prime cDNA synthesis. The libraries were either screened by hybridization with the MIPP cDNA probe or by nested PCR. All screens were unsuccessful at isolating a MIPP cDNA.

Rapid amplification of cDNA ends (5' RACE) was also used to amplify the 5' ends of the MIPP transcripts found in mouse mammary carcinomas. Methods involving addition of 5' homopolymer tails to cDNA, addition of either single- or double-stranded adapters to the 5' end of cDNA, and circularization of cDNA followed by inversion of opposite facing primers by digestion with a restriction endonuclease were all attempted. For each technique, many variations in cDNA synthesis and PCR conditions were tried. However, the results varied from obtaining no amplification products whatsoever, to artefacts which proved to be unrelated to MIPP upon sequence analysis.

The kelch family of proteins can be divided into two sub-families: those which contain a BTB/POZ protein/protein interaction domain, and those which do not. By virtue of its kelch repeats, the putative MIPP protein was deemed a member of the kelch family. However, since only a fragment of the MIPP mRNAs had been cloned, the division to which it belonged was not known. Therefore, a degenerate oligonucleotide complementing conserved BTB/POZ sequences was used to amplify cDNA from a MIPP-expressing mouse mammary tumor by PCR. A cDNA of about 994 bp was amplified, cloned and sequenced. The cDNA has an open reading frame (orf) continuous with that of the previously described 3' end. The sum of known MIPP cDNA sequences is 1823 bp, leaving approximately 200 bp and 3.6 kb of unknown sequences in the 2.2 and 5.6 kb transcripts, respectively (assuming a poly-A tail of 200 nucleotides).

Using the SMART oligonucleotide in a modified version of 5' RACE, the entire 5' end of the 2.2 kb MIPP transcript was amplified from a mouse mammary tumor's RNA. The cDNA has an open reading frame of about 1.7kb that potentially codes for a 556 amino acid protein with a theoretical MW of 67kDa. ProfileScan

(http://ulrec3.unil.ch/) was used to search for known domains in the putative MIPP protein. The highest scoring match was to the BTB domain. A probe from the 5' end of the 2.2 kb transcript (including the BTB domain) hybridized to 2.2, 3.5, and 5.6 kb RNAs from a D2 mouse mammary tumor. The 3.5 kb species may be from a related gene which contains a BTB domain but not kelch repeats since it is not detected with a probe from the 3' end of MIPP transcripts. Many such genes are identified when BLAST is used to search GenBank against the 2.2kb cDNA sequence.

Several sets of primers and PCR conditions were tried before the RACE technique worked. The successful conditions involved uneven annealing temperatures in the first round of PCR, and the use of cDNA primed with a MIPP-specific oligonucleotide. These experiments underscore the difficulty encountered in cloning the 5' ends of MIPP transcripts, and raise the possibility that they possess some property that is resistant to reverse transcription and/or PCR. One possible problem might be high GC-content, but even when the RACE was specifically tailored for GC-rich sequences, no products were obtained. Thus, the 5' end of the 2.2kb cDNA, but not the 5.6kb cDNA, has been cloned. The difficulty encountered in cloning the 5' end of MIPP RNAs may be related to the situation described by Hernandez *et al.* (1997) regarding another kelch-related protein, ENC-1. In adult brain, a 4.5 kb ENC-1 transcript is expressed, but only the 3' end (2.4 kb) could be cloned. The 2.4 kb clone had a large reading frame with a stop codon in an area upstream from the translation start site. It was concluded that the rest of the 5' end must be an untranslated region. Perhaps this suggests that the 5.6 kb MIPP transcript has a long 5' untranslated region which might have implications on the levels of MIPP protein.

It is our hypothesis that the MIPP protein's function involves binding to actin, thereby contributing to neoplastic progression when ectopically expressed. To address this question, a His_6 eptiope tag was added to the 1.7kb MIPP orf by high fidelity PCR, and the orf was cloned into the pcDNA3.1 expression vector. The MIPP cDNA was then transfected into the EL12 mouse mammary epithelial cell line. TransFast (Promega), a cationic lipid reagent, was used, and conditions were optimized with a luciferase assay. Single colonies of cells were isolated and screened for recombinant and endogenous MIPP expression by RT-PCR. Two out of four clones appeared to express the recombinant MIPP 1.7kb orf, but Northern blotting revealed that none of the clones really expressed the cDNA. The transfections were repeated after transferring the cDNA to a vector utilizing the SV40 promoter. The luciferase gene from Promega's pGL3 control vector was replaced with the epitope-tagged MIPP cDNA (this construct will be called pGL3M). However, we again failed to isolate cells expressing MIPP. Finally, we transfected pGL3M into A1N4 human mammary epithelial cells and have obtained stable pools of transfectants which express the MIPP cDNA by Northern blotting. The stable transfectants are now being used in growth, invasion, motility, and tumorigenicity assays to determine whether MIPP has transformed the cells. These experiments should determine whether MIPP can act as an oncogene.

To study MIPP protein, the His_6-tagged 1.7 kb MIPP orf was expressed in E. coli using the pProEx-HT vector, (Gibco/BRL, Grand Island, NY). To purify recombinant MIPP protein (rMIPP) for antiserum production, immobilized metal affinity chromatography (IMAC) was used as a first step. The IMAC-purified protein was run on a preparative SDS polyacrylamide gel and transferred to PVDF. The membrane was stained with a reversible total protein stain (GenoTech), and a thin slice was cut from the membrane lenthwise. This strip was then probed with an anti-His_6 monoclonal antibody (Boerhinger-Mannheim). The strip was lined up with the rest of the blot, and bands reacting with the antibody were excised. The excised bands were homogenized in DMSO, dried, and resuspended in PBS/0.1% Tween-20 before injection. Two rabbitts were immunized against rMIPP. Both antisera were shown to be highly specific against rMIPP by ELISA and Western blotting. By Western blotting, three bands (45, 54, and 70 kDa) were detected in placenta, and a single band (70kDa) was detected in a D2 mouse mammary tumor and in the MOD D2 mouse mammary tumor cell line. No protein was detected in normal lactating mammary gland or in EL12 normal mouse mammary epithelial cells. Since only one protein is seen in mammary tumor cells and tissues despite their expression of two transcripts, our results forward the hypothesis that the 5.6kb transcript may contain a long 5' untranslated region.

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To determine whether MIPP binds to microfilaments, the kelch repeat domain (C-terminal half) of the MIPP protein (rMIPP-KRD) was expressed in E. coli and purified by IMAC. The purified rMIPP-KRD was used in an actin co-sedimentation assay. We showed that rMIPP-KRD, but not recombinant chloramphenicol acetyltransferase, sedimented with actin filaments. Furthermore, in co-immunoprecipitation studies, our anti-MIPP antiserum immunoprecipitates actin, and an anti-actin monoclonal antibody (Sigma, St. Louis, MO) brings down MIPP from MOD mouse mammary tumor cell lysates. Although MIPP is clearly capable of binding actin, immunofluorescence staining with anti-MIPP serum did not give a microfilament-like pattern.

Instead, the staining appears to be associated with the endoplasmic reticulum (ER). Dual staining with fluorescent concanavalin-A (an ER-specific stain in most cell types) is now being done to confirm this association. This opens up the possiblilty that MIPP either directly or indirectly mediates an interaction between the ER and the actin cytoskeleton. Such associations have been reported in other systems (Boevink et al., 1998; Tabb et al., 1998).

The human homolog of MIPP, IPP, has recently been cloned and shown to co-sediment with actin (Kim et al., 1999). The two proteins are over 90% identical. Although MIPP expression was a common feature of many BALB/c mammary carcinomas, we were unable to detect IPP expression in human breast cancer cell lines. We used RT-PCR to look for IPP expression in the following cell lines: HBL-100, HMEC, 184-A1N4, 184-A1N4-TH, MDA-231, T47D, and MCF-7. Mouse placenta was included as a positive control because the primers were designed to amplify both the mouse and human cDNAs. The lack of IPP expression in any human breast cell line tested makes it unlikely that the gene is directly involved in breast cancer.

Regarding the Statement of Work (SOW)
Task number one, which was essentially to determine whether the LTR promotes MIPP transcription in the mouse mammary tumors, has been accomplished. However, alternative methods to those outlined in the SOW were used. Tasks two and three are to determine whether MIPP is an oncogene in mouse mammary carcinogenesis and to determine the function of MIPP protein(s), respectively. These tasks depend upon the cloning of the tumor-associated MIPP transcripts, which presented the most difficulty in this research. The 2.2 kb transcript was cloned after much trouble, but the 5' end of the 5.6 kb transcript was unobtainable. However, there is data to suggest that this 5' end is an untranslated region, making it more or less irrelevant to the initial characterization of the MIPP protein. Progress has been made in accomplishing task four, analyzing IPP in human breast cancers, as IPP expression was not detected in any human breast cancer cell lines analyzed. Therefore, analysis of IPP expression in breast cancer tissues has not been a high priority.

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Key Research Accomplishments
- all MIPP mRNAs share a common 3' end
- the IAP LTR is not the promoter for 2.2, 4.4, or 5.6 kb MIPP transcripts
- cloned the 2.2 kb transcript (has 1.7 kb orf)
- MIPP protein has an N-terminal BTB/POZ and a C-terminal kelch domain (with six repeats)
- expressed MIPP protein in bacterial system
- raised antiserum against recombinant MIPP protein
- detected one protein band in mouse mammary tumors on Western blot with anti-MIPP serum
- ER-like pattern when mouse mammary tumor cells were stained with anti-MIPP serum
- obtained stably-transfected A1N4 cells expressing MIPP 1.7 kb orf
- recombinant MIPP protein co-sediments with actin filaments
- actin and MIPP co-immunoprecipitate

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Reportable Outcomes

- 1999 Gordon Conference on Mammary Gland Biology
  Poster Presentation

- 1998 Chautauqua Breast Cancer Symposium
  Poster Presentation;
  second place in poster competition

Abstract

A solo long terminal repeat (LTR) of an endogenous retrovirus-like element (intracisternal A particle) present in the mouse MIPP gene promotes placenta-specific expression of a 1.2 kb message. This transcript is a truncated form of 2.2 and 4.4 kb MIPP transcripts also found in placental tissue. We have previously shown that mouse mammary preneoplasias and carcinomas of several etiologies ectopically express 2.2 and 5.6 kb MIPP-related mRNAs. We determined that the 3' ends of all MIPP transcripts, which are homologous to the kelch repeat motif, have the same sequence. RT-PCR and 5' RACE techniques were employed to clone the 5' end of the 2.2 kb transcript. Sequence analysis revealed a 1.7 kb open reading frame with an N-terminal BTB/POZ protein/protein interaction domain plus a total of six C-terminal kelch repeats. Similar structure in known or suspected actin-binding kelch family members suggested that the MIPP protein(s) may be actin-binding. Using recombinant proteins expressed in a bacterial system, we showed that MIPP co-sediments with actin filaments in vitro. Preliminary results with an antiserum raised against the recombinant MIPP protein indicate that only one MIPP protein, about 70 kDa, is translated in mouse mammary tumor cells, despite the expression of two transcripts. We are currently transfecting the MIPP cDNA into normal mouse mammary epithelial cells for transformation studies. These experiments will determine whether MIPP can contribute to the process of mammary tumorigenesis.

Abstract

A solo long terminal repeat (LTR) of a defective endogenous retrovirus (intracisternal A particle) present in the mouse MIPP gene promotes placenta-specific expression of a 1.2 kb message. This transcript is a truncated form of 2.2 and 4.4 kb MIPP transcripts also found in placental tissue. We have previously shown that mouse mammary preneoplasias and carcinomas of several etiologies ectopically express 2.2 and 5.6 kb MIPP-related mRNAs. The solo LTR was absent from both mammary tumor-expressed MIPP messages, suggesting that LTR activation is not the mechanism responsible for their expression in the tumors. The remainder of the 5' end of the 2.2 kb transcript was cloned by RACE using the SMART oligonucleotide (Clontech). Sequence analysis revealed the presence of a full BTB/POZ domain plus two more kelch repeats directly upstream of the four known repeats. Similar structure in known or suspected actin-binding kelch family members suggests that the MIPP protein(s) may be actin-binding. Therefore, an epitope-tagged recombinant MIPP protein is being expressed in EL12 mouse mammary epithelial cells to determine its subcellular location and interaction with actin by immunofluorescence and immunoprecipitation. Efforts are also focused on cloning the 5.6 kb mammary carcinoma-specific transcript, with the goal of determining if MIPP functions as an oncogene.